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Herpes Simplex Virus Type 1 Latency in the Murine Nervous System Is Associated with Oxidative Damage to Neurons

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The pathological consequences of herpes simplex virus type 1 (HSV-1) latency in the nervous system are not well understood. To determine whether acute and latent HSV-1 infections of the nervous system are associated with oxidative damage, mice were inoculated with HSV-1 by the corneal route, and the extent of viral infection and oxidative damage in trigeminal ganglia and brain was assessed at 7, 90, and 220 days after inoculation. Abundant HSV-1 protein expression in the nervous system was observed in neurons and non-neuronal cells at 7 days after inoculation, consistent with viral replication and spread through the trigeminal and olfactory systems. Acute HSV-1 infection was associated with focal, neuronal and non-neuronal 4-hydroxy-2-nonenal- and 8-hydroxyguanosine-specific immunoreactivity, indicating oxidative damage. Rare HSV-1 antigen-positive cells were observed at 90 and 220 days after inoculation; however, widespread HSV-1 latency-associated transcript expression was detected, consistent with latent HSV-1 infection in the nervous system. HSV-1 latency was detected predominantly in the trigeminal ganglia, brainstem, olfactory bulbs, and temporal cortex. Latent HSV-1 infection was associated with focal chronic inflammation and consistently detectable evidence of oxidative damage involving primarily neurons. These results indicate that both acute and latent HSV-1 infections in the murine nervous system are associated with oxidative damage.

INTRODUCTION

Most adult humans have nervous system infections with herpes simplex virus type 1 (HSV-1) due to the capacity of the virus to establish latency in neurons following primary infection (reviewed in Roizman and Sears, 1996; Whitley, 1996; Johnson and Valyi-Nagy, 1998). During latent HSV-1 infection, viral DNA is present in neuronal nuclei, and the latency-associated transcripts (LATs) are the only abundant viral RNAs expressed. Viral proteins and infectious virions usually are not detectable. Latent infections with HSV-1 are asymptomatic; however, reactivation of viral replication, which can be symptomatic or clinically inapparent, does occur in many virus carriers. These reactivations most often involve peripheral tissues such as the face and cornea, whereas reactivations associated with neurologically significant disease, such as encephalitis, are rare (reviewed in Whitley, 1996; Johnson and Valyi-Nagy, 1998). The source of virus for all reactivation events is thought to consist of neurons in either the peripheral nervous system (PNS) or the central nervous system (CNS) (reviewed in Roizman and Sears, 1996).

Studies using animal models of HSV-1 pathogenesis indicate that neurologically inapparent viral reactivations occur spontaneously in the nervous system and can be experimentally induced in latently infected animals (Tullo *et al.,* 1982; Willey *et al.,* 1984; Hill, 1985; Laycock *et al.,* 1991; Sawtell and Thompson, 1992; Bloom *et al.,* 1994; Fawl *et al.,* 1996). These reactivations usually involve only a small number of cells in the nervous system and rarely progress to encephalitis (Sawtell and Thompson, 1992; Bloom *et al.,* 1994; Fawl *et al.,* 1996; Shimeld *et al.,* 1996). Observations made using reverse transcription–polymerase chain reaction to detect viral transcripts in neural tissues of mice latently infected with HSV-1 suggest that HSV-1 latency in neurons is associated with constant, low-level expression of viral immediate-early genes in addition to the abundant expression of the LATs (Kramer and Coen, 1995). Perhaps due to limited, repeated reactivation events or constant, low-level viral antigen expression, HSV-1 latent infection in the nervous system of laboratory animals is associated with low-grade, persistent, chronic inflammation and persistently elevated levels of cytokines (Cantin *et al.,* 1995; Liu *et al.,* 1996), growth-associated protein 43 (Martin *et al.,* 1996), and inducible nitric oxide synthetase (Koprowski *et al.,* 1993; Meyding-Lamade *et al.,* 1998). Long-term treatment of latently infected animals with the antiherpesvirus drug acyclovir results in normalization of cytokine levels, which suggests that viral DNA replication and protein

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expression are responsible for the induction of cytokines during HSV-1 latency in the nervous system (Halford *et al.,* 1997). These findings raise the possibility that HSV-1 latency is associated with low-grade injury to the nervous system in the absence of overt encephalitis.

In this study, we used the well-characterized murine model of HSV-1 neuropathogenesis (Knotts *et al.,* 1974) to investigate whether latent HSV-1 infection is associated with oxidative damage to neurons. Injury mediated by free radicals, particularly by partially reduced oxygen species, is an important common pathway of such varied pathological processes as radiation damage, chemical and traumatic injury, ischemia-reperfusion injury, inflammatory damage, neurodegenerative diseases, and cellular aging (Knight, 1995; Love and Jenner, 1999; Toyokuni, 1999). Free radicals are extremely reactive and readily enter into reactions with macromolecules, particularly membrane lipids and nucleic acids. Importantly, free radicals can cause lipid peroxidation, an autocatalytic process that damages lipid-containing structures and yields reactive byproducts, primarily 4-hydroxy-2-nonenal (HNE), which can covalently modify and damage cellular macromolecules. Methods available for the detection of free radical-induced damage include immunohistochemical localization of either protein adducts from the chemically reactive lipid peroxidation by-product HNE or 8-hydroxyguanosine (8OHG), the most abundant adduct formed on nucleic acids by hydroxyl radical attack (Yin *et al.,* 1995; Montine *et al.,* 1998; Zhang *et al.,* 1999). Using HNE- and 8OHG-specific antisera, we show that HSV-1 latency in the murine nervous system is associated with oxidative damage to neurons.

RESULTS

Infection of mice with HSV-1

To determine whether acute and latent infections of mice with HSV-1 are associated with oxidative damage to neural tissues, BALB/c mice were inoculated on the cornea with either 1×10^5 plaque-forming units (PFU) of HSV-1 strain 17^+ (Brown *et al.,* 1973) or sterile tissue-culture medium as a mock-infection control (Deatly *et al.,* 1987; Valyi-Nagy *et al.,* 1991a,b). Twenty-two percent of the HSV-1-infected mice demonstrated signs of encephalitis (agitation or paralysis), and 13% of the animals died within 3 weeks of infection. Animals surviving beyond 3 weeks of virus inoculation demonstrated no signs of encephalitis and showed normal development. Groups of five or six randomly chosen HSV-1-infected and mock-infected mice were euthanized at 7, 90, and 220 days postinfection (p.i.). These timepoints were chosen on the basis of previous studies using the same experimental conditions that demonstrated acute HSV-1 infection can be detected at 7 days p.i. in the trigeminal ganglia (TG) and CNS of mice and that latent infection is established by 30 days p.i. in the TG and CNS of mice surviving acute infection (Valyi-Nagy *et al.,* 1991a,b, 1992). TG and brain were aseptically removed from euthanized animals, and tissues were fixed in paraformaldehyde-lysine-periodate fixative, embedded in paraffin, and sectioned (6 μm). For each animal, sections of the TG, brainstem, cerebrum, and olfactory bulbs were prepared and analyzed by immunohistochemical staining for HSV-1 antigen and *in situ* hybridization for LAT expression to document acute and latent HSV-1 infection (Fig. 1, Table 1).

In animals euthanized 7 days after HSV-1 infection, viral proteins were detected in many CNS sections by immunostaining (Fig. 1A, Table 1), consistent with widespread replication of HSV-1 as has been previously reported (Knotts *et al.,* 1974; Valyi-Nagy *et al.,* 1991a,b; Wagner and Bloom, 1997). Most of the viral protein immunoreactivity was observed in the brainstem, hypothalamus, thalamus, and olfactory bulbs. Viral protein immunoreactivity was less abundant in the TG, which is consistent with previous observations that HSV-1 replication at this site is nearly resolved by 7 days p.i. (Valyi-Nagy *et al.,* 1991a, 1992). HSV-1 proteins were detected in cells identified by morphological criteria as both neurons and non-neuronal cells. In TG and CNS tissues obtained from animals euthanized at 90 and 220 days p.i., no HSV-1 antigen expression was detected in CNS tissues, and occasional HSV-1-antigen-expressing non-neuronal cells were detected in some TG tissues (Table 1). LAT RNA expression was detected by *in situ* hybridization in TG and CNS tissues of HSV-1-infected mice euthanized at 7, 90, and 220 p.i. (Fig. 1F, Table 1). Because LAT expression occurs during both productive and latent infection and HSV-1 protein expression occurs only during productive infection, these findings indicate that mice examined at 7 days p.i. were productively infected with HSV-1, whereas those examined at 90 and 220 days p.i. were latently infected with the virus.

Inflammation associated with HSV-1 infection in the murine nervous system

Sections of TG and CNS tissues stained with hematoxylin and eosin at 7, 90, and 220 days after HSV-1 infection revealed inflammatory changes consistent with previous reports (Knotts *et al.,* 1974; Stroop *et al.,* 1984; Liu *et al.,* 1996). Tissue sections from HSV-1-infected animals euthanized at 7 days p.i. showed perivascular and parenchymal inflammatory infiltrates in the TG and CNS consisting primarily of mononuclear cells, in addition to focal tissue necrosis and neuronophagia (Fig. 2A and data not shown). All 10 TG and 5 brains examined at 7 days p.i. demonstrated inflammatory changes. Focal chronic inflammation was noted in 4 of 5 TG and 1 of 5 brains at 90 days p.i. and in 8 of 10 TG and 1 of 5 brains at 220 days p.i. Inflammatory changes were not detected in the nervous system of mock-infected animals. Mononuclear inflammatory cells were observed surrounding

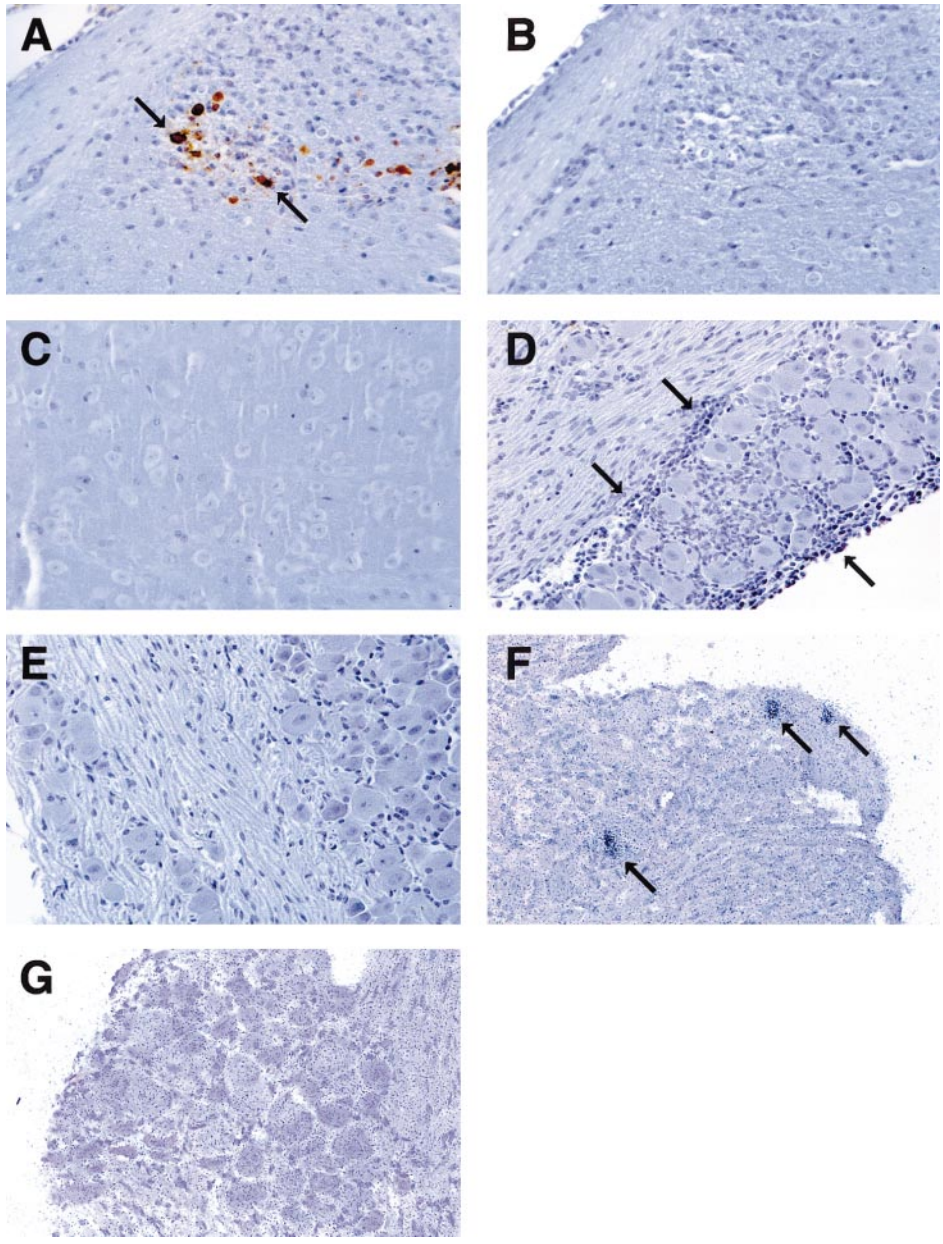

FIG. 1. HSV-1 protein immunoreactivity and LAT expression in the nervous system of HSV-1-infected or mock-infected mice. (A) HSV-1 proteins in the hypothalamus 7 days after inoculation with HSV-1. Arrows indicate two neurons immunoreactive for HSV-1 proteins. (B) Staining for HSV-1 proteins without HSV-1-specific antiserum in the immunohistochemical reaction as a specificity control. (C) HSV-1 proteins in the thalamus 7 days after mock infection. HSV-1 proteins in TG 220 days after inoculation with (D) HSV-1 or (E) mock infection. Arrows in panel D indicate areas of mononuclear inflammatory infiltrates. LAT expression in TG 220 days after inoculation with (F) HSV-1 or (G) mock infection. Arrows in panel F indicate neurons positive for LAT expression.

morphologically intact neurons in the TG of latently infected animals, and occasional neuronophagia was noted (Fig. 2C). Chronic inflammatory foci in the CNS were observed in the brainstem, olfactory bulbs, temporal and parietal cortical areas, and leptomeninges (Fig. 2E and data not shown). Immunohistochemical staining for lymphocyte common antigen (LCA) and human alveolar macrophage (HAM) 56 confirmed the presence of focal, mixed lymphocytic and macrophagic/microglial infiltrates, respectively, in tissues latently infected with HSV-1 (Figs. 2G and 2I). These findings indicate that acute and latent HSV-1 infections of mice are associated with inflammatory changes in CNS and PNS tissues.

Acute and latent HSV-1 infections in the murine nervous system are associated with HNE-protein adducts

To determine whether acute and latent HSV-1 infections in the murine nervous system are associated with

TABLE 1

Mean Number of Cells per Histologic Section Demonstrating HSV-1 Immunoreactivity and HSV-1 LAT Expression in the Trigeminal Ganglia (TG), Brainstem (BS), Cerebrum (CER), and Olfactory Bulbs (OB) of Mice after Corneal Inoculation with HSV-1

				No. of positive cells per section (mean ± S.D.)					
				HSV-1 IR[a]			HSV-1 LAT		
Infection	Time after infection (d)	No. of animals studied	Site	No. of sections studied	Neurons[b]	Other cells[c]	No. of sections studied	Neurons	Other cells
Mock	7, 9, 220	12	TG	26	0	0	13	0	0
			BS	18	0	0	9	0	0
			CER	24	0	0	12	0	0
			OB	14	0	0	7	0	0
HSV-1	7	5	TG	12	2.0 ± 2.1	2.0 ± 2.4	6	6.0 ± 3.4	2.0 ± 1.0
			BS	10	16.0 ± 8.7	11.4 ± 5.6	5	18.6 ± 11.8	6.6 ± 4.4
			CER	20	12.6 ± 7.2	8.1 ± 5.2	15	16.3 ± 16.4	5.8 ± 5.0
			OB	6	0	13.0 ± 16.9	6	0	13.3 ± 18.5
HSV-1	90	5	TG	12	0	0.2 ± 0.2	6	18.5 ± 7.0	0.8 ± 0.9
			BS	10	0	0	5	0.4 ± 0.8	0
			CER	10	0	0	5	0	0
			OB	8	0	0	4	0	4.2 ± 7.5
HSV-1	220	5	TG	10	0	0.1 ± 0.3	10	23.9 ± 10.8	2.6 ± 3.2
			BS	10	0	0	5	0.6 ± 1.3	0
			CER	15	0	0	15	1.8 ± 5.3	0.8 ± 3.3
			OB	4	0	0	4	0	0.2 ± 0.5

[a] IR, immunoreactivity.
[b] Neurons, morphologically identified as neurons; total number per section in TG 629 ± 130, in CNS >5000.
[c] Other cells, cells lacking definitive morphological features of neurons; total number per section in TG >2500, in CNS >5000.

oxidative damage, TG and CNS tissues were examined by immunohistochemistry using an antiserum specific for reducible protein adducts of HNE, a specific and highly cytotoxic product of lipid peroxidation (Montine *et al.,* 1998). Reducible HNE-protein adduct immunostaining in tissues from acutely infected animals revealed numerous immunoreactive neurons and non-neuronal cells (Fig. 3A, Table 2). Immunoreactive neurons were observed primarily in inflamed areas of the TG, brainstem, hypothalamus, and thalamus (Fig. 3A). Tissues from mock-infected animals showed occasional immunoreactive neurons in the TG; however, numbers of immunoreactive neurons in HSV-1-infected animals were significantly greater than those in mock-infected animals ($P < 0.01$) (Table 2). HNE-immunoreactive non-neuronal cells were not observed in tissues from mock-infected animals, and no HNE-immunoreactive cells were detected in the CNS of uninfected mice (Fig. 3C, Table 2). Although HNE-immunoreactive cells were readily detectable in HSV-1-infected TG and brain tissues, the relative frequency of these cells in the sections analyzed was low: <3% in the TG and <1% in the CNS (Table 2). Reducible HNE-protein adduct immunostaining demonstrated oxidative damage in PNS and CNS tissues of latently infected mice in excess of that observed in mock-infected mice (Figs. 3D and 3E, Table 2). The TG of HSV-1-infected animals euthanized at 90 and 220 days p.i. contained significantly greater numbers of HNE-positive neurons and non-neuronal cells than those in the TG of uninfected animals ($P < 0.02$) (Table 2). HNE-positive cells in tissues from infected mice were both neurons and non-neuronal cells, and these cells were most abundant in chronically inflamed areas (Figs. 3D and 4D). The extent of HNE immunoreactivity in the TG of uninfected mice at 90 and 220 days after mock infection was similar to that observed at 7 days after mock infection (Table 2). The CNS of some HSV-1-infected animals contained low numbers of HNE-positive neurons and non-neuronal cells (Table 2). HNE-positive cells in the CNS were most often in areas demonstrating chronic inflammation in the olfactory bulbs, temporal cortex, and brainstem. In contrast, the CNS of mock-infected animals contained no HNE-positive cells at any time-point ($P < 0.01$) (Table 2). The relative frequency of HNE-positive cells in tissues latently infected with HSV-1 was <3% and <1% among neurons in the TG and CNS, respectively, and ≪1% at both sites among non-neuronal cells (Table 2).

HNE immunoreactivity colocalizes with HSV-1 infection in the murine nervous system

The relationship of HNE immunoreactivity to HSV-1 protein and LAT expression in acutely and latently infected tissues, respectively, was assessed by immunohistochemistry and *in situ* hybridization performed on serial sections (Fig. 4). In tissues from acutely infected

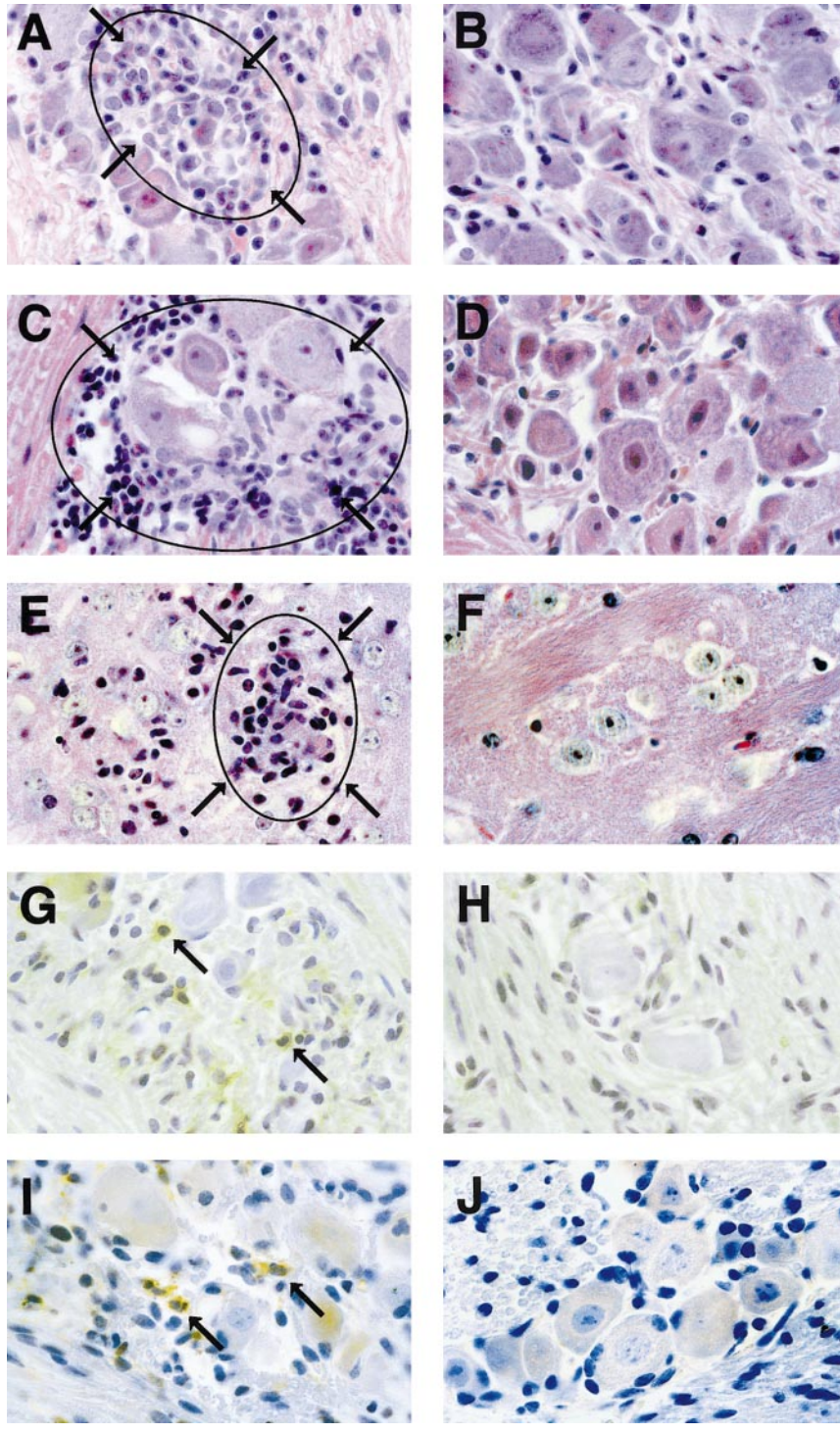

FIG. 2. Inflammation in the nervous system of HSV-1-infected mice. (A–F) Hematoxylin and eosin staining of PNS and CNS tissues of mice infected with HSV-1. TG 7 days after inoculation with (A) HSV-1 or (B) mock infection. TG 220 days after inoculation with (C) HSV-1 or (D) mock infection. (E) Frontal cortex 90 days after HSV-1 infection. (F) Striatum 90 days after mock infection. Encircled areas in panels A, C, and E indicate foci of mononuclear inflammatory infiltrates in HSV-1-infected tissues. (G–J) Immunohistochemical detection of LCA and HAM-56 in the TG of mice infected with HSV-1. LCA-immunoreactivity (arrows) in TG 90 days after inoculation with (G) HSV-1 or (H) mock infection. HAM-56 immunoreactivity (arrows) in TG 90 days after inoculation with (I) HSV-1 or (J) mock infection.

mice at 7 days p.i., HNE-positive cells were found to stain either positively or negatively for HSV-1 proteins (Figs. 4A and 4B). HNE-positive, HSV-1-negative cells were located in inflamed foci in close proximity to cells expressing HSV-1 proteins. Similarly, in latently infected tissues, HNE-positive cells were found to be either LAT positive

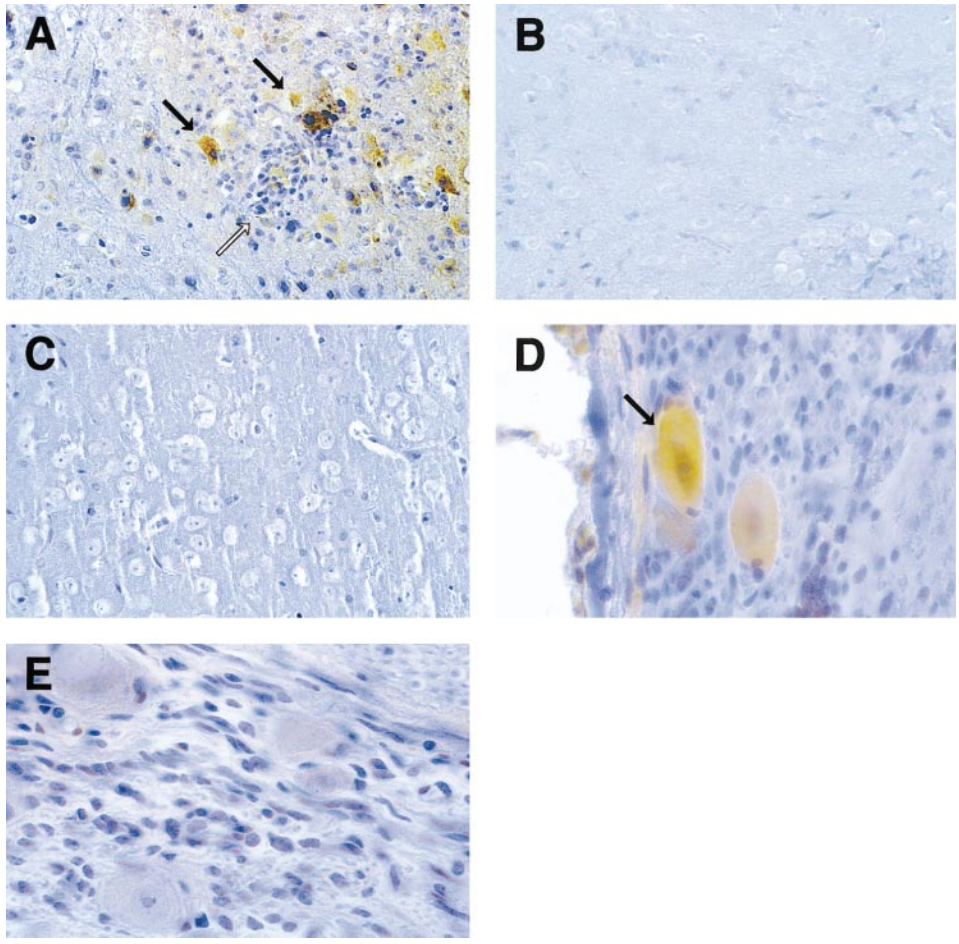

FIG. 3. HNE-protein adduct immunoreactivity in the nervous system of HSV-1-infected or mock-infected mice using an antiserum specific for reducible HNE-protein adducts. (A) Brainstem 7 days after inoculation with HSV-1. Closed arrows indicate representative neurons in the section immunoreactive for HNE-protein adducts. The open arrow indicates a collection of mononuclear inflammatory cells. (B) HNE-protein adduct immunoreactivity in the cerebral cortex 7 days after inoculation with HSV-1 in the absence of borohydrate pretreatment as a specificity control. (C) Cerebral cortex 7 days after mock infection. TG 220 days after inoculation with (D) HSV-1 or (E) mock infection. Arrow in panel D indicates a neuron immunoreactive for HNE protein-adducts.

or LAT negative; HNE-positive, LAT-negative cells were located adjacent to inflamed foci in proximity to LAT-positive neurons (Figs. 4C and 4D). Therefore, HNE immunoreactivity localizes to sites of acute and latent HSV-1 infection in the murine nervous system but is not restricted to HSV-1-infected cells.

Acute and latent HSV-1 infections in the murine nervous system are associated with the generation of 8OHG

To provide additional evidence of oxidative damage in association with HSV-1 infection, tissue sections from HSV-infected and mock-infected mice were stained with a monoclonal antibody specific for 8OHG, the most abundant adduct formed on RNA or DNA by hydroxyl radical attack (Yin *et al.,* 1995; Zhang *et al.,* 1999). Brain tissue of mice euthanized 7 days following infection with HSV-1 demonstrated 8OHG-specific immunoreactivity in numerous neurons and non-neuronal cells (Fig. 5A, Table 2). Positive cells were observed primarily in inflamed areas of the brainstem, thalamus, and hypothalamus. 8OHG immunoreactivity was mostly cytoplasmic, consistent with previous reports of 8OHG immunohistochemical reactivity in human neurodegenerative diseases (Nunomura *et al.,* 1999; Zhang *et al.,* 1999). Minimal immunoreactivity was observed in tissues pretreated with DNase and RNase, which confirms that the staining detected in these experiments was associated with nucleic acid (Fig. 5E). Brain tissue of HSV-1-infected mice euthanized at 7 days p.i. demonstrated significantly greater numbers of 8OHG-immunoreactive cells than brain tissue of mock-infected mice ($P < 0.01$) (Table 2). Cells staining for 8OHG in tissues from uninfected animals consisted primarily of scattered hypothalamic neurons. 8OHG-specific immunohistochemistry performed on TG tissues from uninfected mice demonstrated weak immunoreactivity of most neurons with

TABLE 2

Mean Number of Cells per Histologic Section Demonstrating Oxidative Damage (HNE and 8OHG Immunoreactivity) and Apoptosis in the Trigeminal Ganglia (TG), Brainstem (BS), Cerebrum (CER), and Olfactory Bulbs (OB) of Mice after Corneal Inoculation with HSV-1

				No. of positive cells per section (mean ± SD)								
				HNE IR[a]			8OHG IR			TUNEL		
Infection	Time after infection (d)	No. of animals studied	Site	No. of sections	Neurons[b]	Other cells[c]	No. of sections	Neurons[b]	Other cells[c]	No. of sections	Neurons[b]	Other cells[c]
Mock	7, 90, 220	12	TG	26	0.9 ± 1.5	0	13	HB[d]	0	26	0	0
			BS	18	0	0	12	0.9 ± 1.6	0	24	0	0
			CER	24	0	0	24	5.4 ± 4.8	0	24	0	0
			OB	14	0	0	ND[e]	ND	ND	24	0	0
HSV-1	7	5	TG	12	5.9 ± 4.0	4.9 ± 3.3	6	HB	3.1 ± 4.6	12	1.1 ± 1.8	24.3 ± 14.9
			BS	10	4.3 ± 3.8	2.4 ± 2.0	10	25.0 ± 17.1	16.1 ± 12.9	10	1.7 ± 1.6	14.8 ± 15.7
			CER	30	5.0 ± 6.0	4.1 ± 4.8	10	23.9 ± 17.9	13.5 ± 17.2	10	1.0 ± 1.6	6.2 ± 8.7
			OB	12	0	10.0 ± 16.3	ND	ND	ND	6	0	13.1 ± 18.5
HSV-1	90	5	TG	12	3.5 ± 3.4	2.0 ± 3.6	ND	ND	ND	12	0.3 ± 0.8	3.3 ± 5.1
			BS	10	0	0	ND	ND	ND	10	0	0
			CER	10	0	0	ND	ND	ND	10	0	0
			OB	8	0	2.0 ± 3.5	ND	ND	ND	8	0	0
HSV-1	220	5	TG	20	3.8 ± 5.8	1.4 ± 2.4	10	HB	5.4 ± 7.7	20	0.4 ± 0.6	1.9 ± 3.1
			BS	10	0.3 ± 0.6	0	10	19.5 ± 22.4	5.8 ± 6.6	10	0	0.6 ± 0.8
			CER	30	2.3 ± 8.3	0.5 ± 2.2	30	11.6 ± 21.4	4.1 ± 8.3	30	0	0.4 ± 0.8
			OB	8	0	0.2 ± 0.7	ND	ND	ND	8	0	0.1 ± 1.0

[a] IR, immunoreactivity.
[b] Neuron, morphologically identified as neurons; total number per section in TG: 629 ± 130, in CNS >5000.
[c] Other cells, cells lacking definitive morphological features of neurons; total number per section in TG >2500, in CNS >5000.
[d] HB, high background staining.
[e] ND, not done.

no associated non-neuronal staining (data not shown). This background staining precluded an evaluation of the effect of HSV-1 infection on 8OHG modification of nucleic acid in the TG.

Immunostaining for 8OHG modification of nucleic acid also revealed evidence of oxidative damage in the CNS during HSV-1 latency. Brain tissue of mice euthanized 220 days following infection with HSV-1 contained significantly greater numbers of 8OHG-immunoreactive cells per section than CNS tissue from uninfected animals ($P < 0.01$) (Table 2). Most of the 8OHG-immunoreactive cells were neurons in inflamed areas of the temporal cortex and brainstem (Fig. 5C). Background staining in the TG precluded an analysis of the effect of HSV-1 latency on 8OHG immunoreactivity at that site (data not shown). These findings confirm our results using HNE-specific immunostaining and indicate that acute and latent HSV-1 infections are associated with oxidative damage to neurons.

Acute and latent HSV-1 infections in the murine nervous system are associated with apoptosis

To determine whether neural tissue damage in acute and latent HSV-1 infection is associated with apoptosis, TG and CNS tissues were examined for DNA strand breaks using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay. TUNEL-positive cells were observed throughout the PNS and CNS of mice acutely infected with HSV-1 and were detected primarily in inflamed areas of the TG, brainstem, hypothalamus, and thalamus (Fig. 6A, Table 2). Morphologically, these cells consisted almost exclusively of non-neuronal cells. In contrast, TUNEL-positive cells were not observed in tissues of mock-infected, control mice (Fig. 6B, Table 2). A low number of TUNEL-positive non-neuronal cells and occasional TUNEL-positive neurons were observed in latently infected PNS tissues (Fig. 6C, Table 2). Occasional TUNEL-positive non-neuronal cells also were noted in the CNS of a latently infected animal; however, TUNEL-positive cells were not observed in tissues of mock-infected, control mice (Fig. 6D, Table 2). Although TUNEL positivity is not specific for apoptosis and can also mark necrotic cells (Charriaut-Marlangue and Ben-Ari, 1995), our findings suggest that apoptosis is a component of tissue injury during acute and latent HSV-1 infection in the murine nervous system. Furthermore, our results suggest that apoptosis in HSV-1-infected tissues is restricted primarily to non-neuronal cells.

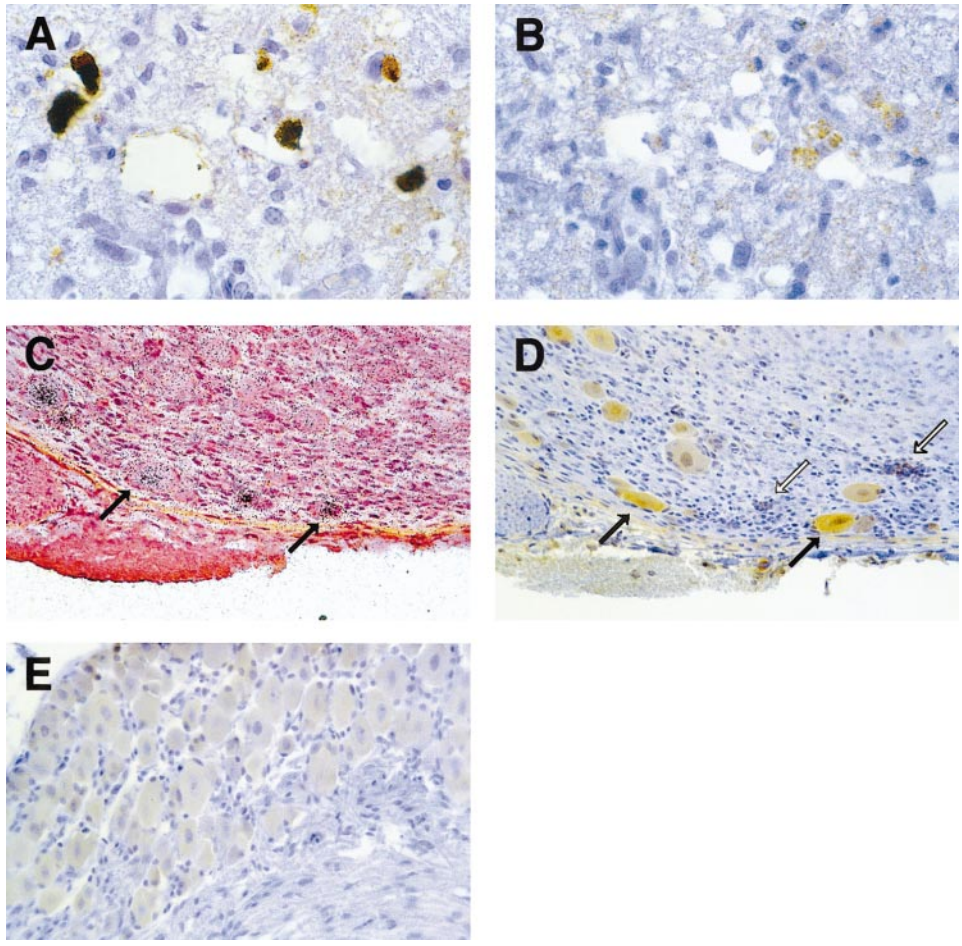

FIG. 4. Localization of HSV-1 protein and LAT expression relative to HNE-protein adduct immunoreactivity in the nervous system of acutely and latently infected mice, respectively. (A) HSV-1 proteins and (B) HNE-protein adduct immunostaining in serial sections of the brainstem 7 days after inoculation with HSV-1. (C) LAT expression and (D) HNE-protein adduct immunostaining in serial sections of TG 220 days after inoculation with HSV-1. (E) HNE-protein adduct immunostaining in TG 220 days after mock infection. Arrows in panel C indicate LAT-positive neurons. Closed arrows in panel D indicate neurons demonstrating HNE-protein adduct immunoreactivity that also express LAT as shown in panel C. Open arrows in panel D indicate areas with dense collections of inflammatory cells.

DISCUSSION

Our findings indicate that both acute and latent HSV-1 infections in the murine nervous system are associated with oxidative damage. In comparison to acute infection, oxidative damage is less extensive during latent infection; nonetheless, it is consistently observed. Oxidative damage during acute HSV-1 infection involves both neurons and non-neuronal cells; however, HSV-1 latency is associated with oxidative damage involving primarily neurons.

Experiments using antisera specific for HNE-protein adducts and 8OHG indicate that oxidative damage is a consequence of acute, productive HSV-1 infection in the nervous system of mice. Oxidative damage during acute HSV-1 infection occurs predominantly in foci of viral replication and is associated with mononuclear cell infiltration. Oxidative stress and oxidative damage are recognized pathological consequences of some viral infections (reviewed in Peterhans, 1997; Akaike *et al.,* 1998; Valyi-Nagy *et al.,* 1999); therefore, our findings were not unanticipated. However, we were surprised by the detection of HNE- and 8OHG-specific immunoreactivity in numerous neurons several months after acute infection. Because little is known about the clearance of HNE-protein adducts and 8OHG in the murine nervous system, it is possible that some or all of the neurons exhibiting evidence of oxidative damage at 90 and 220 days p.i. were damaged during the acute phase of infection. However, the detection of chronic inflammation during latency suggests that at least some component of the oxidative damage detected at late times after inoculation is due to increased oxidative stress during latent infection.

What accounts for the oxidative damage during HSV-1 infection in the murine nervous system? It is possible that low-level expression of viral genes other than the LATs during latency (Kosz-Vnenchak *et al.,* 1993; Kramer and Coen, 1995), either directly through the production of

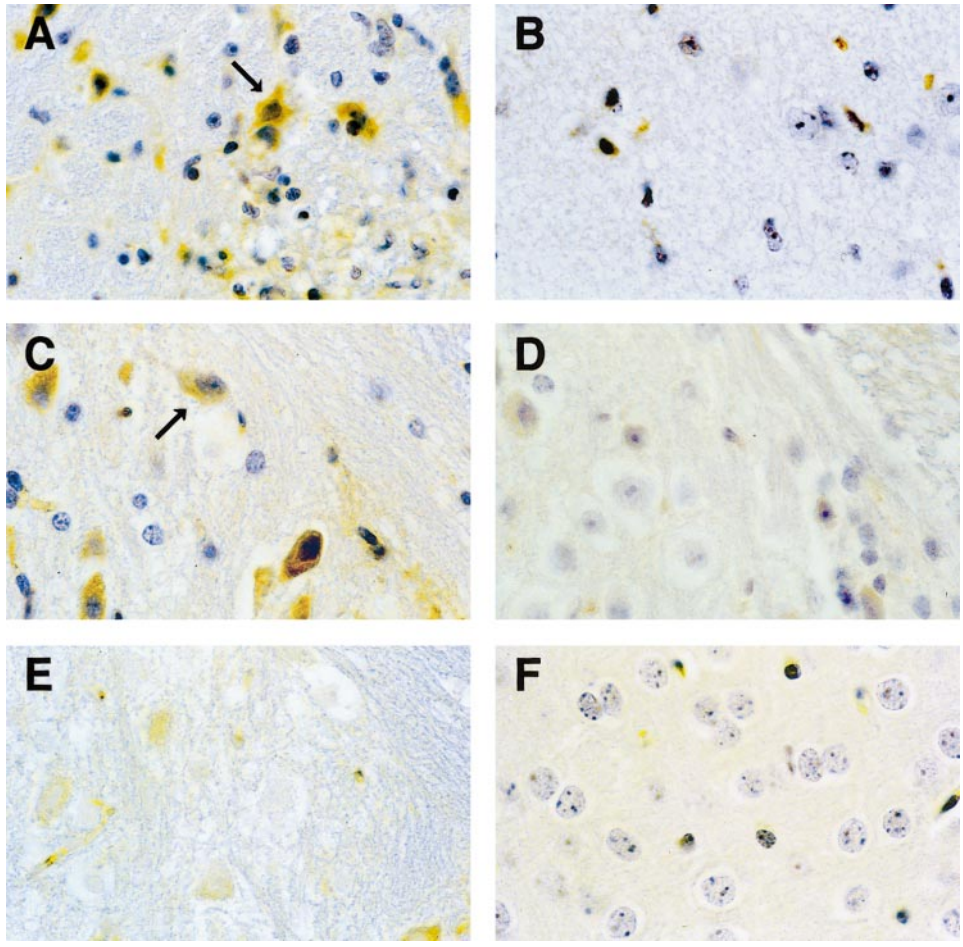

FIG. 5. 8OHG immunoreactivity in the CNS of HSV-1-infected and mock-infected mice. Brainstem 7 days after inoculation with (A) HSV-1 or (B) mock infection. (C) Brainstem 220 days after HSV-1 infection. Staining for 8OHG (D) without primary antibody or (E) following DNase and RNase treatment as specificity controls. (F) Cerebral cortex 220 days after mock infection. Arrows in panels A and C indicate 8OHG-immunoreactive neurons in HSV-1-infected tissues.

cytotoxic viral proteins or indirectly through the deleterious effects of inflammatory mediators, contributes to the tissue damage detected in the nervous system of mice latently infected with HSV-1. It is also possible that LAT RNAs or latent viral genomes contribute to the neuropathology observed in tissues from latently infected mice, perhaps by altering cellular gene expression or affecting the activity of the RNA-dependent protein kinase, PKR. Colocalization of the oxidative damage marker HNE with HSV-1 protein and LAT expression in acutely and latently infected tissues, respectively, suggests that the oxidative damage associated with acute and latent HSV-1 infection affects both infected and uninfected cells. Therefore, it is likely that at least some component of the oxidative damage associated with HSV-1 infection is mediated by either viral or host factors capable of injuring uninfected cells. Regardless of the proximate cause of the oxidative damage detected at late times p.i., our findings clearly show that latent HSV-1 infection in the murine nervous system is associated with oxidative neural injury.

Along with the detection of oxidative damage in latently infected tissues, results reported here suggest that apoptotic cell death is also associated with latent HSV-1 infection in the nervous system of mice. Although cells undergoing necrosis can be detected by TUNEL staining (Charriaut-Marlangue and Ben-Ari, 1995), our findings suggest that apoptosis is a component of the tissue injury observed during both acute and latent HSV-1 infection in the murine nervous system. Acute HSV-1 infection has been previously reported to induce apoptosis of neurons in the TG in rabbits (Perng *et al.,* 2000). However, to our knowledge, apoptosis during latent HSV-1 infection has not been previously described. Because DNA fragmentation and cellular apoptotic bodies are unlikely to be detectable several months after an initiating stimulus, it is probable that the fragmented DNA detected by TUNEL assay at 90 and 220 days p.i. did not result from cell death during the acute phase of HSV-1 infection. Instead, our results suggest that non-neuronal cells, as well as occasional neurons, undergo apoptosis during latent HSV-1 infection. The types of non-neuronal cells undergoing apoptosis are not appar-

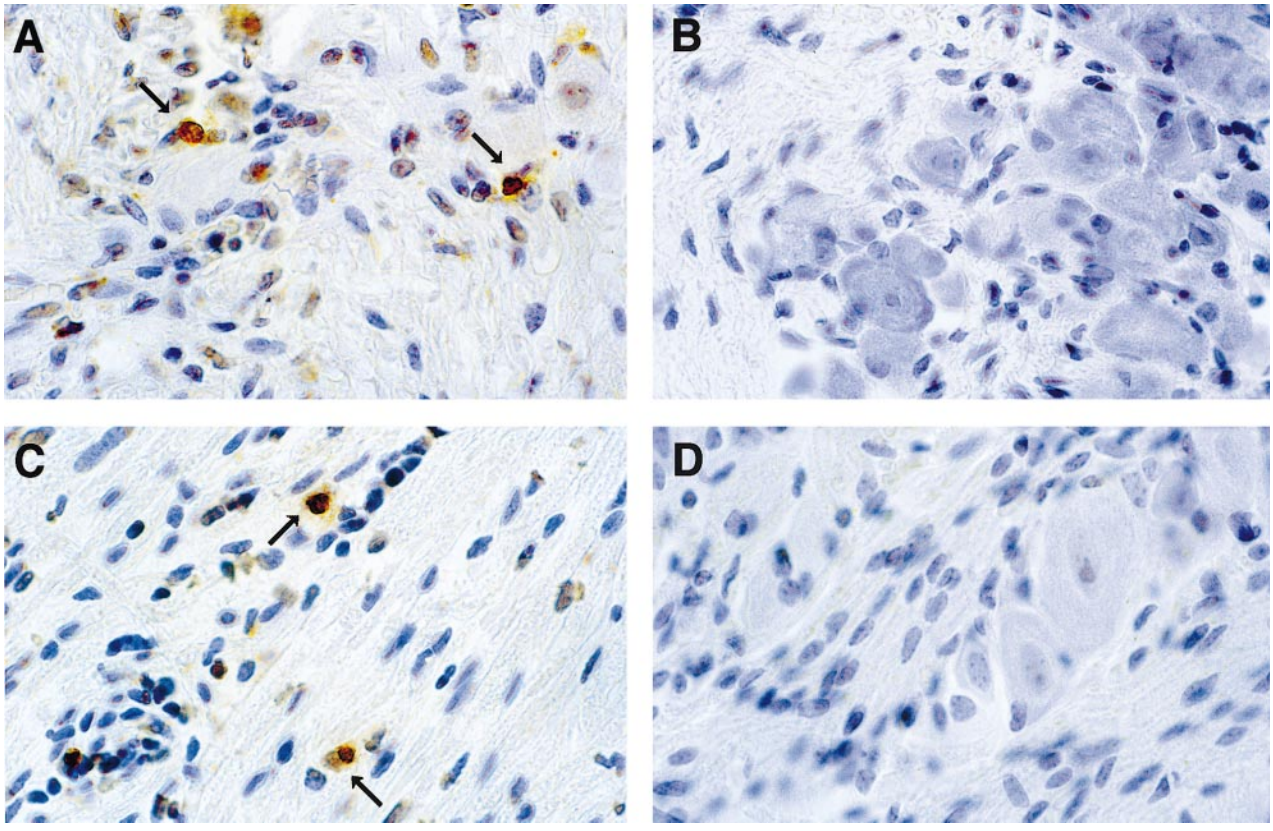

FIG. 6. TUNEL-positive cells in the nervous system of HSV-1-infected or mock-infected mice. Sections were analyzed by TUNEL assay to detect apoptosis-associated DNA strand breaks. TG 7 days after inoculation with (A) HSV-1 or (B) mock infection. TG 220 days after inoculation with (C) HSV-1 or (D) mock infection. Arrows in panels A and C indicate TUNEL-positive cells.

ent from our study, but it is likely that inflammatory cells constitute the majority of these cells.

The distribution of TUNEL-positive cells in acutely and latently infected neural tissues suggests that the cellular DNA fragmentation detected in these experiments is associated with apoptosis rather than HSV-1 DNA. In acutely infected CNS and PNS tissues, TUNEL-positive cells were predominantly non-neuronal cells, despite the fact that numerous neurons stained positively for HSV-1 proteins. This finding suggests that HSV-1 replication is not sufficient to cause TUNEL positivity. In latently infected TG, numerous LAT-positive neurons were detected in the absence of significant TUNEL staining, which provides evidence that latent HSV-1 DNA is not a source of DNA termini for labeling with digoxigenin-conjugated dUTP. Thus, the TUNEL staining observed in our study is not likely to result from the labeling of HSV-1 DNA present in infected cells.

Although we found no evidence of massive tissue injury associated with HSV-1 latency in these experiments, our findings raise the possibility that the modest tissue damage related to HSV-1 latency is neurologically significant during the life span of the host. Further research using genetically defined strains of mice, HSV-1 mutant strains, and longer observation periods will be required to provide insight into the potential clinical significance of the tissue damage caused by HSV-1 latency in the murine nervous system.

Findings reported here raise the possibility that HSV-1 latency in the human nervous system is associated with oxidative injury. The pathogenesis of several human neurodegenerative diseases, most notably Alzheimer's disease, is thought to involve exposure to as-yet-unidentified environmental factors in persons with an appropriate genetic predisposition (reviewed in Esiri *et al.,* 1996). It remains to be determined whether HSV-1 latency in the nervous system contributes to human neurodegenerative disease. Due to the high prevalence of latent HSV-1 infections in the CNS of adult humans (Liedtke *et al.,* 1993; Baringer and Pisani, 1994; Sanders *et al.,* 1996), it is likely that latent HSV-1 infection per se does not cause significant nervous system damage. However, tissue damage associated with HSV-1 latency may serve as a pathogenic trigger for some forms of neurodegenerative disease when certain viral virulence and host susceptibility requirements are met. To explore this possibility, our ongoing work is directed at identifying viral and host determinants of the extent of tissue damage associated with HSV-1 latency. These studies will lead to a better understanding of the clinical significance and pathological consequences of HSV-1 latency in the nervous system.

MATERIALS AND METHODS

Virus stocks

HSV-1 strain 17^+ (Brown *et al.,* 1973) was obtained from Dr. Nigel Fraser (University of Pennsylvania, Philadelphia, PA).

Inoculation of mice

Four- to 6-week-old female BALB/c mice (Harlan, Indianapolis, IN) were inoculated with 1×10^5 PFU of HSV-1 17^+ per eye after corneal scarification using Metofen anesthesia as previously described (Deatly *et al.,* 1987; Valyi-Nagy *et al.,* 1991a,b). Control mice were inoculated with an equivalent volume of tissue culture medium using the same technique (mock infection).

Recovery of nervous system tissues

Mice were euthanized by cervical dislocation at 7, 90, and 220 days after inoculation, and TG and brain were aseptically removed. Tissues were fixed in paraformaldehyde-lysine-periodate fixate, embedded in paraffin, and sectioned (6 μm). For CNS tissues, coronal sections of brainstem, cerebrum, and olfactory bulbs were prepared and analyzed for each animal.

Immunohistochemistry

Tissue sections for immunohistochemistry were deparaffinized with xylene and rehydrated through a series of graded ethanols. Endogenous peroxide was quenched in a 0.3% H_2O_2–methanol bath followed by several washes with PBS. Before staining, binding of secondary antibodies and conjugates was blocked by appropriate reagents provided by each manufacturer. HSV-1 antigens were detected using a 1:1000 dilution of a polyclonal anti-HSV-1 antiserum raised in a rabbit (DAKO, Carpinteria, CA). Tissue sections were incubated with primary antibody at 43°C for 32 min before the addition of biotinylated anti-rabbit immunoglobulin secondary antibody, avidin-horseradish peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris–HCl (pH 7.4) and 0.025% H_2O_2 as a chromogen (Ventana Medical Systems, Tucson, AZ).

Reducible protein adducts of HNE were detected using a 1:100 dilution of HNE-specific antiserum 672 (Montine *et al.,* 1998) and an automated immunostainer. Prior to immunohistochemical detection of HNE, sections were incubated with 10 mM sodium borohydrate for 30 min as antiserum 672 reacts with the stable, reduced Michael adducts of HNE that are formed on reduction with borohydrate (Montine *et al.,* 1998). Sections were washed three times with PBS and then incubated with antiserum 672 at 43°C for 32 min. Tissue sections treated with borohydrate were incubated with a 1:100 dilution of preimmune serum as a negative control. As an additional negative control, sections were incubated with primary antiserum alone without prior treatment with borohydrate. Experimental and control sections were incubated with biotinylated anti-rabbit immunoglobulin secondary antibody, avidin-horseradish peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris–HCl (pH 7.4) and 0.025% H_2O_2.

8OHG was detected using a 1:50 dilution of an 8OHG-specific murine monoclonal antibody (Yin *et al.,* 1995; Zhang *et al.,* 1999). Sections were first incubated at 43°C for 4 min in 0.5 U/ml of protease I (Ventana) and then incubated at room temperature for 1 h with the 8OHG-specific antibody. Immunohistochemical staining was performed using the HistoMouse Kit (Zymed, San Francisco, CA), which is designed specifically to reduce nonspecific, mouse–mouse interactions. Diaminobenzidine tetrahydrochloride was used as a chromogen. Controls for each experiment included sections processed without primary antibody and sections pretreated with 5 μg/ml DNase-free RNase (Boehringer-Mannheim, Indianapolis, IN), 10 U/ml RNase-free DNase (Boehringer-Mannheim), and 10 U/ml S1 DNase (Boehringer-Mannheim) in PBS or PBS alone at 37°C for 1 h.

LCA and HAM-56 were detected using murine monoclonal anti-LCA and anti-HAM-56 antibodies (DAKO), respectively, and the HistoMouse kit with diaminobenzidine tetrahydrochloride as a chromogen.

Cells containing detectable HSV-1 proteins, HNE-protein adducts, and 8OHG were quantified in each section examined, and the mean number of immunoreactive cells for each antibody and time point was determined. Immunoreactive cells were identified morphologically as neurons based on large nuclei, prominent nucleoli, and extensive cytoplasm; non-neuronal cells lacked these morphological features.

In situ hybridization for HSV-1-LAT gene expression

In situ hybridization was performed using a nick-translated ^{35}S-labeled DNA probe specific for HSV-1 LAT (*Bst*2–*Bst*2 fragment) (Valyi-Nagy *et al.,* 1992) according to previously described techniques (Deatly *et al.,* 1987; Valyi-Nagy *et al.,* 1991a,b). The probe used in these experiments had a specific activity of $>10^8$ cpm/μg. LAT-positive cells, morphologically identified as either neurons or non-neuronal cells, were quantified in each section examined, and the mean number of positive cells per section was determined for each timepoint.

Detection of apoptosis

Apoptotic cell death in TG and brain of HSV-1-infected and mock-infected mice was determined by the detection of apoptosis-associated DNA strand breaks in tissue sections using a TUNEL assay (ApopTag Plus; Onco, Gaithersburg, MD). Cells demonstrating *in situ* end-la-

beling were quantified in each examined TG and brain section, and the mean number of positive cells per section was determined for each timepoint.

Data analysis

Tissue sections processed for immunohistochemistry, *in situ* hybridization, and TUNEL staining were reviewed by an observer blinded to the nature of the infection (HSV-1 or mock) and the time after inoculation. Results were analyzed using a one-tailed *t* test.

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